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Installation Restoration Research Program

Implementation Guidance for Determining Suitability of Microorganisms for Explosives Degradation

by Douglas Gunnison, Herbert L. Fredrickson, David B. Ringelberg, WES

Edward J. Perkins, AScI Corporation

Prepared for Headquarters, U.S. Army Corps of Engineers

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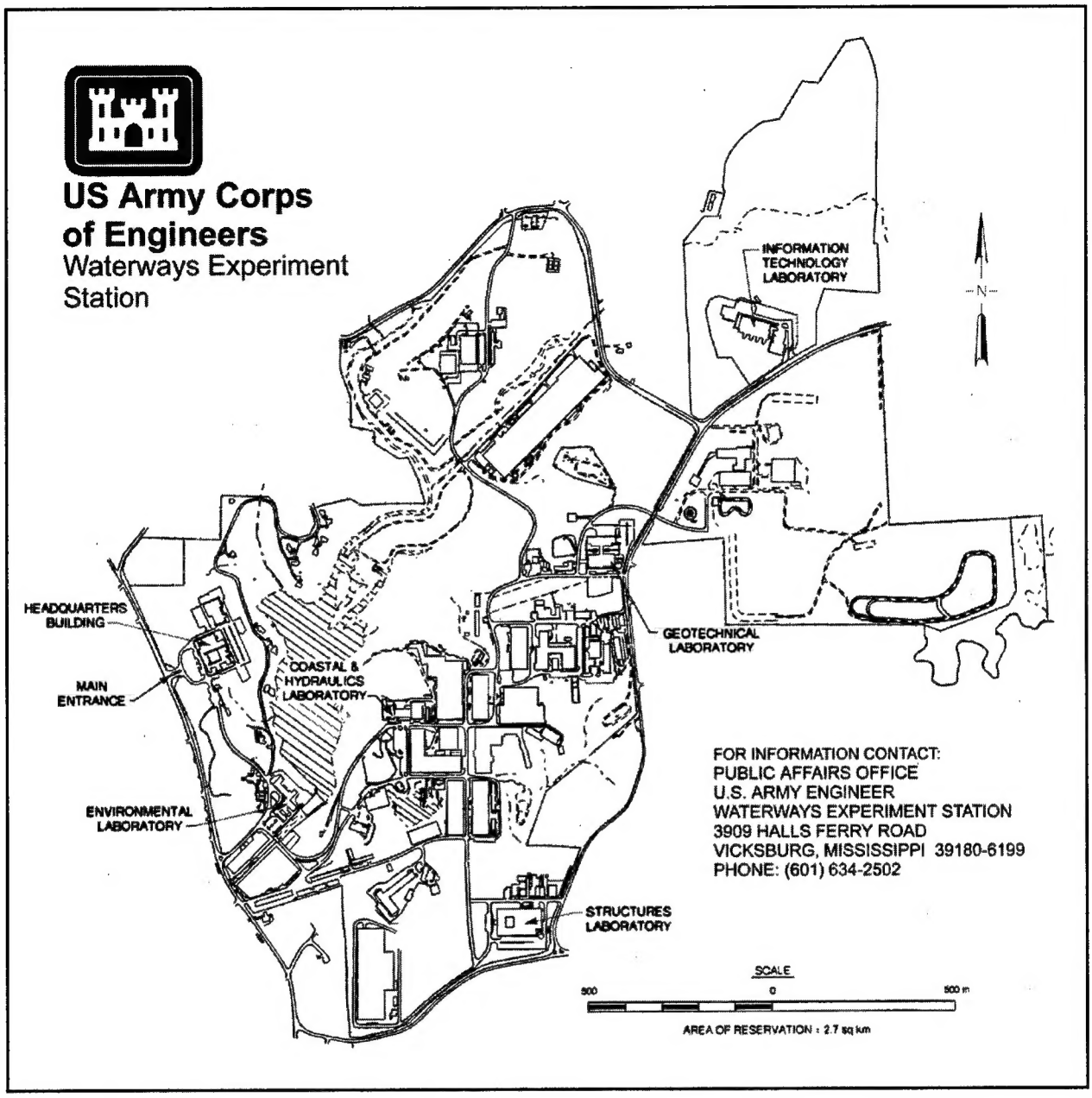

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Contents

Preface

The work reported herein was conducted by the U.S. Army Engineer Waterways Experiment Station (WES), Vicksburg, MS, for Headquarters, U.S. Army Corps of Engineers (HQUSACE). Funding was provided by the HQUSACE Installation Restoration Research Program (IRRP), Work Unit AH68-BR001, "Basic and Supporting Research - Microbiology and Degradation of Explosives." Dr. Clem Myer was the IRRP Coordinator at the Directorate of Research and Development, HQUSACE. The IRRP Program Manager was Dr. M. John Cullinane, Director, Environmental Information Analysis Center, Environmental Laboratory (EL), U.S. Army Engineer Waterways Experiment Station (WES), Vicksburg, MS, a complex of five laboratories of the Engineer Research and Development Center (ERDC).

This report was prepared by Drs. Douglas Gunnison, Herbert L. Frederickson, and David B. Ringelberg, Ecosystem Processes and Effects Division, EL; and Edward J. Perkins, AScI Corporation.

The work was conducted under the direct supervision of Dr. Robert H. Kennedy, Chief, Ecosystem Processes and Effects Branch; and under the general supervision of Dr. Richard E. Price, Environmental Processes and Effects Division, EL; and Dr. John W. Keeley, Acting Director, EL.

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1 Introduction

Background

Nearly 17,000 sites on Department of Defense (DoD) installations require environmental cleanup. Many of these sites include soil, sediment, and groundwater contaminated with the explosives 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX or cyclonite), or octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). Methods for remediating explosives-contaminated soil materials require neutralization or detoxification to prevent harmful impacts on macro- and microorganisms (Selivanovskaya et al. 1987; Bumpus and Tatarko 1994; Fuller and Manning 1997a, 1997b). Areas containing high levels of explosives contamination require intensive measures in order to attain acceptable decontamination levels (i.e., incineration). The bulk of subsurface explosives contamination, however, occurs at low concentrations. Use of intensive cleanup measures is expensive and requires levels of handling too demanding for practical use in the cleanup of widespread low-level contamination. Exploitation of microorganisms to degrade explosives offers an opportunity to attain cost-effective treatment of these compounds. For biotreatment to work, procedures must be available to identify the form and function of those microorganisms having the best potential for success. Here form refers to the taxonomic identity of the microorganisms, while function refers to the role of the microorganisms in the degradation process. In identifying the function of the microorganisms, it is vital to determine the pathways that must be supported during the biotreatment process and provide the environmental conditions most critical to sustaining their activity.

Many investigations have demonstrated that microorganisms are able to degrade explosives (Regen and Crawford 1994; Vanderberg, Perry, and Unkefer 1995; Monpas et al. 1997; Young, Unkefer, and Ogden, 1997; Young et al. 1997). In addition, basic studies on the metabolism and physiology of TNT-, 2,4- and 2,6-dinitrotoluene (2,4- and 2,6-DNT), RDX-, and HMX-degrading microorganisms are common in the literature (Spanggord et al. 1984; McCormick, Cornell, and Kaplan 1981; Regen and Crawford 1994; Kitts, Cunningham, and Unkefer 1994; Binks, Nicklin, and Bruce 1995; Vanderberg, Perry, and Unkefer 1995; Monpas et al. 1997; Young, Unkefer, and Ogden 1997; Young et al. 1997). However, these experimental studies do not provide the basic information required to evaluate explosives-degrading microorganisms in

soil and sediment for potential use in research investigations and biological treatment. Since the environmental and microbiological factors regulating contaminant removal vary extensively for different soils and sediments, it is essential that this basic information be readily obtainable.

Purpose and Scope

Microbial destruction of explosives in soil and sediment is an important component of biological treatment measures designed to clean up explosives-contaminated matrices. To this end, it is vital to demonstrate that micro-organisms used for biological treatment possess vigorous activity against explosives and produce acceptable, environmentally benign end products. This report provides guidance on how to detect, isolate, and perform physiological, biochemical, genetic, and environmental characterizations of TNT-, RDX-, and HMX-degrading microorganisms.

2 Presence and Characteristics of Explosives Degradation

The following methods provide the investigator with the tools necessary to (a) establish the presence of microbial explosives-destroying activity, (b) determine the rate and extent of explosives degradation, and (c) assess the explosives degradation products released by the microorganisms present in the soil or sediment sample.

Screening for Active Explosives-Degrading Microorganisms

Complete details of the procedure for screening of soil and sediment for microbial activity against TNT are provided in Gunnison et al. (1993). The same procedures may be used for RDX and HMX. Briefly, soil or sediment samples are obtained from sites contaminated by explosives storage, disposal, and/or manufacturing. Several subsamples are pretreated with potential cometabolites to "prime" their microbial inhabitants for explosives degradation. Untreated and pretreated soil or sediment samples are placed onto mineral salts agar coated with a crystalline lawn of TNT, RDX, or HMX and incubated. It is recommended that now replicate plates be incubated under aerobic, anaerobic, and microaerophilic (conducted in a closed container having a nitrogen gas atmosphere with an oxygen content of approximately 3 percent) conditions as well. Plates are read after 2 and 4 weeks of incubation at 30 °C and scored for the presence or absence of growth and interactions with the explosive, as indicated by the formation of a clear zone surrounding the soil clump and release of colored metabolites into the agar (Figure 1). Positive plates exhibit growth outward from the soil clump and clearing of the explosive in areas immediately surrounding the growth, with or without color change.

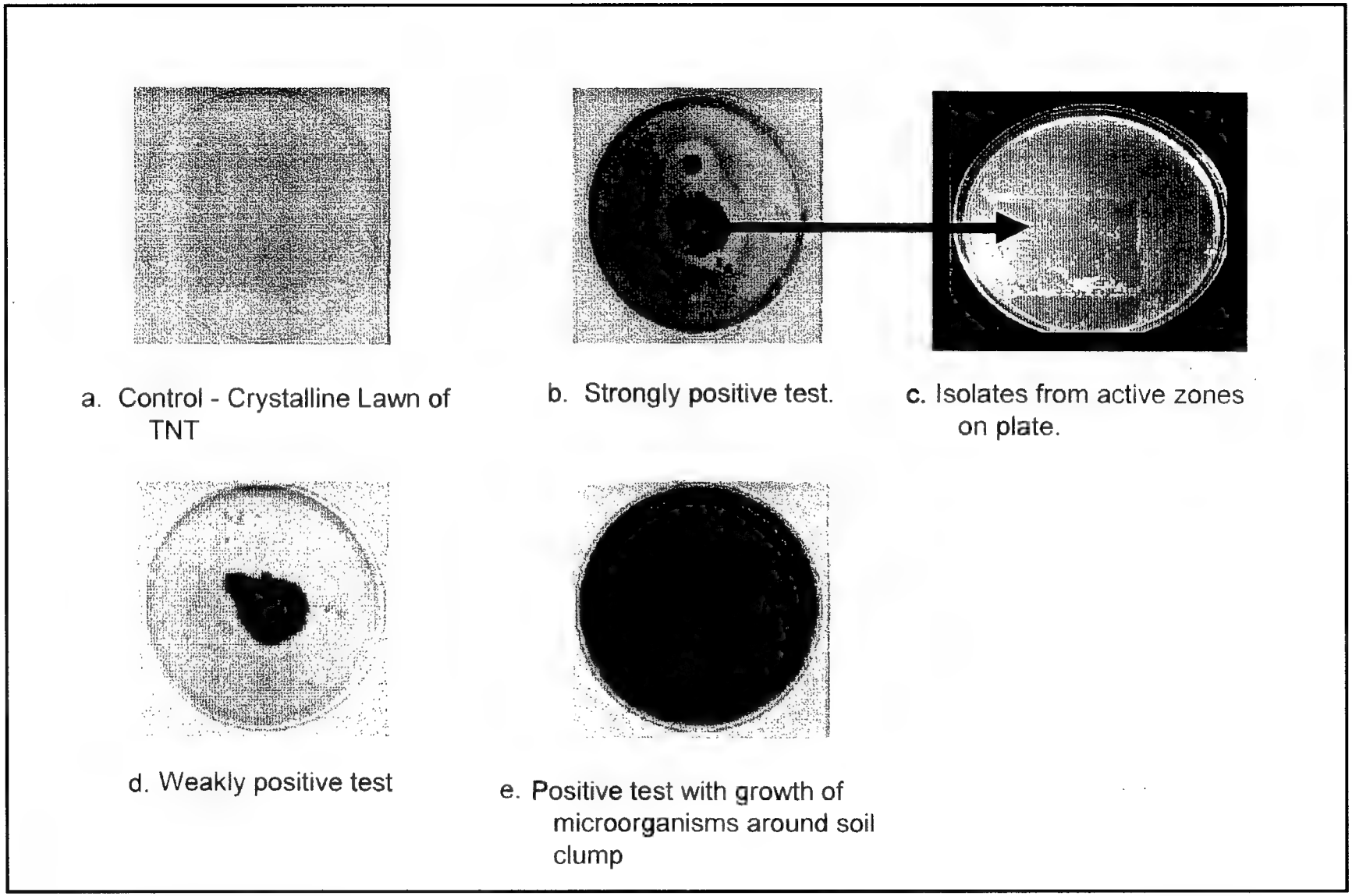

Figure 1. Screening and isolation procedure for identifying soils with microorganisms having the ability to degrade TNT (modified from Gunnison et al. 1993)

Radiorespirometry for Evaluation of Explosives Mineralization

Soils from plates exhibiting positive activity in the screening procedure are tested with radiorespirometry using biometer flasks (either standard biometer or Gledhill types, Bellco Glass, Inc., Vineland, NJ). These tests are conducted to detect active explosives-mineralizing microorganisms in a soil or sediment sample. Mineralization refers to the ability of microorganisms to degrade the explosive compound to its basic inorganic components (CO_2, or CH_4, nitrate or ammonia, etc.). Briefly, the procedures for the biometer flasks are as follows (details are available in general form in Schmidt and Scow (1997), and the specific details are given in Gunnison et al. (1993)). First, the dry weight equivalent of 9 g of moist or wet soils or sediments mixed with sterile distilled water to form 30 percent (weight per volume (w/v)) slurries or the microorganisms collected from a 1-L water sample concentrated on a 0.2-μL micropore filter are resuspended in 30 mL of the filter-sterilized sample water. Microorganisms in the biometers are then challenged, with 0.2 μCi of ^{14}C-acetate, ^{14}C-TNT, ^{14}C-RDX, or ^{14}C-HMX in the biometer flasks, and the 1N potassium hydroxide (KOH) traps on the biometers are monitored for the rate and extent of $^{14}CO_2$ production (Figure 2). $^{14}CO_2$ released by acetate mineralization over a period of 2-5 days indicates the overall viability of the microorganisms present in the soil, sediment, or water sample. Minimal mineralization levels of 25 percent or higher indicate that active microbial populations are present, while levels of 35 percent or higher in the same time frame indicate that very active (robust) microbial populations are present and healthy. Degradation of radiocarbon-labeled RDX or TNT to $^{14}CO_2$ levels above the 2 to 3 percent impurities in the compounds over 28 to 30 days indicates the microorganisms present are able to mineralize these compounds (Figure 3). To provide sufficient material for chemical analysis, studies can also be conducted with cold (nonradioactive) TNT added to biometer flasks at levels of 10 to 100 mg TNT•kg^{-1} soil (10 to 50 mg TNT•L^{-1} of water). These biometers are run in parallel with their radioactive counterparts.

Product Extraction and Identification

At the end of an assay with either cold or radiolabeled TNT, or both, the microcosm phases (water, soil, and KOH traps) are separated and analyzed to determine the location of the residual radioactivity and identify the products, if separable. Procedures for performing this with unlabeled products resulting from incubation with cold TNT are given in Gunnison et al. (1993). The same procedures are used for radiolabeled samples, but must be conducted employing Nuclear Regulatory Commission (NRC)-approved procedures for use of radiotracers, and the analysis should be conducted on instrumentation dedicated to radiotracer investigations. Briefly, these procedures are as follows.

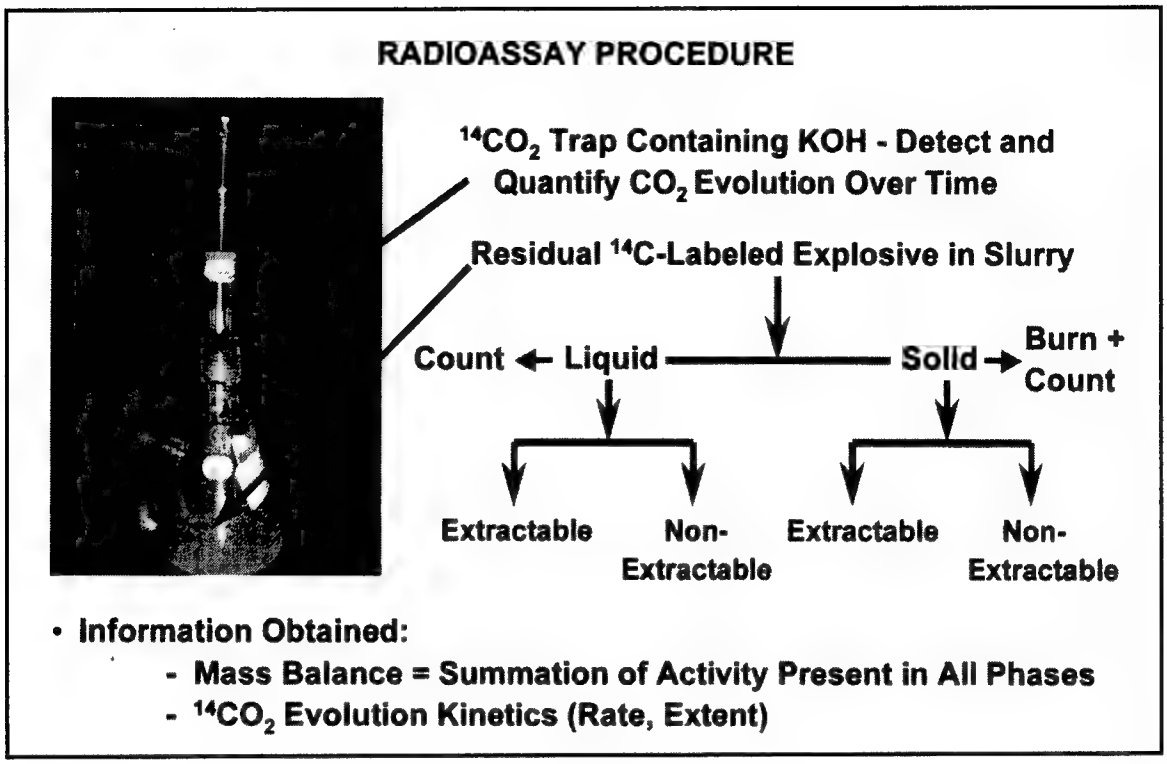

Figure 2. Major components of the biometer flask. Indicated are the location of the KOH trap for radiolabeled CO_2 and the extraction scheme for assessment of the location and nature of the explosives and explosives degradation products at the end of the incubation. Details are provided in the text.

Degradation products for identification and quantification are isolated and identified using a Bligh-Dyer extraction (White et al. 1979), followed by sonication and evaporation to dryness under nitrogen, redissolution in methylene chloride, and passage through a prewashed silica gel column. The explosives are moved through the column with methylene chloride, redried under a nitrogen atmosphere, and dissolved in a 50:50 mixture of acetonitrile and water. Compounds are separated and identified using U.S. Environmental Protection Agency (USEPA) method 8330 (USEPA 1994) employing a high-performance liquid chromatograph (HPLC) equipped with either a photodiode array detector (PDAD) or a radiomatic detector for cold or radiolabeled explosives, respectively. Compounds are separated on the HPLC with a mobile phase of ammonium chloride, methanol, and butanol under isocratic conditions. The identity and quantity of individual explosive degradation products are determined by comparison with known explosives standards run through the same analytical system.

Results of the $^{14}CO_2$ evolution portion of the radiorespirometry assay indicate whether or not soil or sediment samples harbor sufficient microbial activity against the explosive to warrant efforts to isolate individual microorganisms or microbial consortia. For the explosives TNT, RDX, and HMX, water, soil, or sediment samples having mineralization activities of at least 10 to 15 percent per gram dry weight (equivalent) of material over an incubation period of 28 to

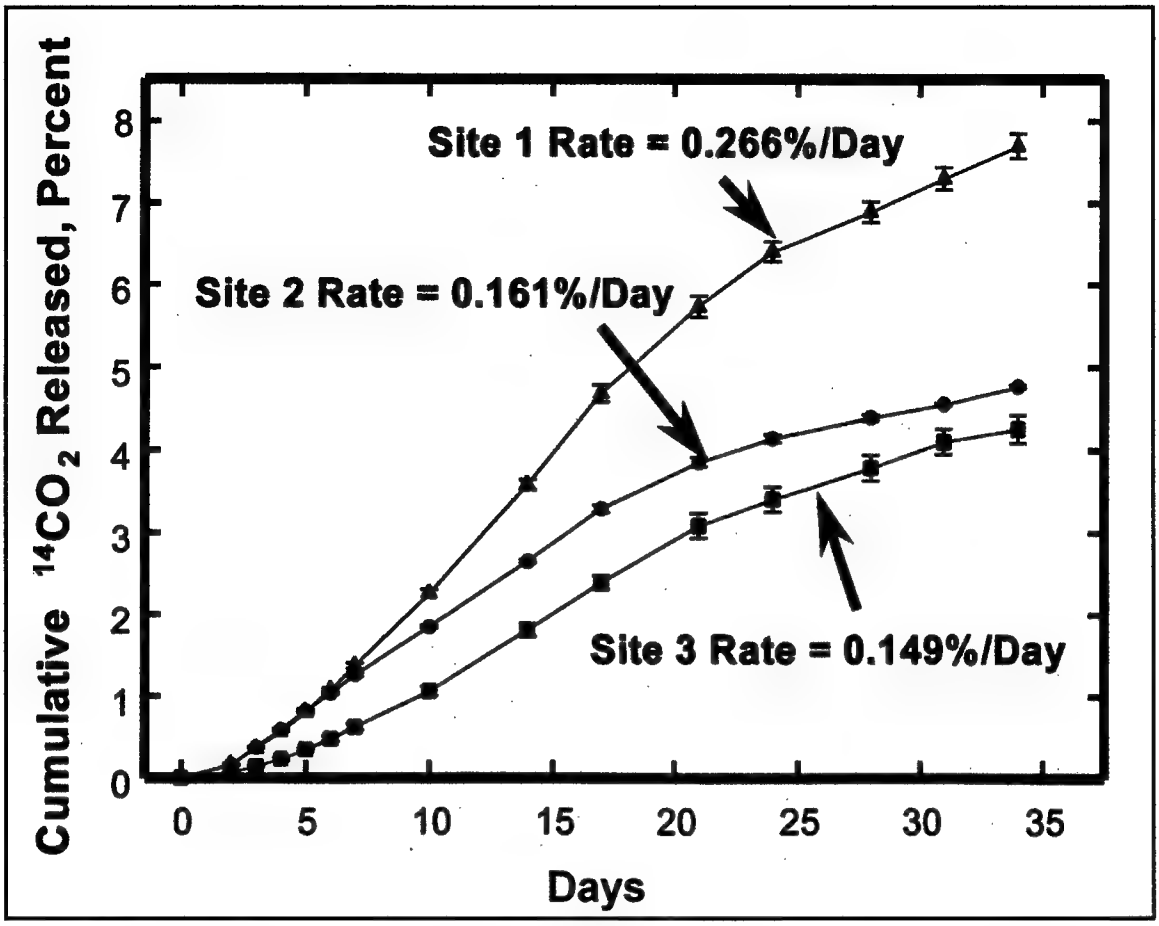

Figure 3. TNT mineralization in surface soils from an Army Ammunition Plant. Rates were derived by running linear regressions on the data for each curve

30 days are acceptable for microbial isolation. Isolation efforts are best spent obtaining microorganisms or microbial mixtures from soil or sediment environments that do not produce and accumulate high levels of mono- and diaminonitrotoluenes and azoxynitrotoluenes, as these are undesirable products.

3 Physiological, Biochemical, and Genetic Properties of Explosives-degrading Microorganisms

Use of the procedures described in Chapter 2 allows the engineer or scientist to detect the presence of microorganisms able to degrade explosives to desirable end products. The isolation and identification procedures and the physiological, biochemical, and genetic characterizations given in Chapter 3 will demonstrate whether or not these isolates utilize degradation pathways amenable to laboratory or biotreatment reactor systems.

Isolation of Explosives-Degrading Microorganisms

Isolation of microorganisms able to degrade explosives is attempted using growth from the screening plates described in Chapter 2, following scoring, or from any soil or sediment having potential explosives-degrading activity. Briefly, M-8 medium solidified with 1.5 percent agar and containing 20 to 50 mg of explosive per liter final volume of the medium, as described in Gunnison et al. (1997), is recommended for isolation work. Other suitable growth media include SGGR broth and agar or MRS agar (Coleman, Nelson, and Duxbury 1998). To increase the opportunity for explosives-degrading microbial isolates to grow, some of the media should include a cometabolite such as sodium benzoate or soluble starch at an approximate concentration of 1 percent. Since large clumps of microbial growth may have zones of widely differing oxidation-reduction status, replicate plates of the inoculated media are incubated under aerobic, anaerobic, and microaerophilic conditions in the dark at 20-25 °C. Plates are examined for growth after 2 and 4 weeks, and colonies showing growth to a diameter of 3 to 5 mm are picked and transferred to fresh medium. Following regrowth on agar, colonies exhibiting the most vigorous growth should be transferred to liquid M-8 medium containing the explosive at the same

concentration as for the solid. For cases where a cometabolite was added to the solid medium, at least two flasks should be used, one with and one without the cometabolite. Flasks are incubated under the same atmosphere as for the plates.

Cultures are checked for purity, and samples of the individual cultures are transferred to biometer flasks containing 30 mL of M-8 salts medium with a 0.2 μCi spike of U-[^{14}C]-TNT and assayed for $^{14}CO_2$ production. Successful mixed or pure cultures will release a minimum of 6 to 10 percent of the radiolabeled carbon initially added as the $^{14}CO_2$ product within 2 weeks. Effective cultures are retained on agar and liquid versions of medium from which they were isolated, but small portions of the liquid cultures are also frozen under 50 percent volume to volume (v:v) glycerol for future use.

Identification of Microbial Isolates/Predominant Microbial Species

Accomplishment of this work depends on the use of pure cultures. If predominant microbial species present in a mixture are to be identified, they should be isolated from the rest of the microorganisms present in the culture.

Microbial isolates in pure culture may be identified using the lipids present in their cell membranes. Briefly, the cells are extracted for lipids using the procedure of Bligh and Dyer, and the lipid fractions are separated and then esterified to create the methyl esters (fatty acid methyl esters or FAMEs). The latter compounds are separated by gas-liquid chromatography. To identify the unknown microorganism(s), the resulting FAME patterns are then compared with FAME patterns for known microorganisms. The lipid identification process can be obtained commercially on a fee per culture basis supplied by several different companies.

To obtain nucleic acids for microbial identification, isolated cells are grown in pure culture, 16S ribosomal deoxyribonucleic acid (16S rDNA) is separated from a subsample of the culture, the DNA is copied by the polymerase chain reaction (PCR), and the DNA is sequenced and analyzed using a DNA sequencer. The identity of the isolated 16S rDNA is obtained through comparison with the 16S rDNA extracts of known microorganisms to identify the unknown sample (Figure 4) (procedure available in Lane 1991). The 16S rDNA identification process can be obtained commercially on a fee per culture basis supplied by several different companies.

Assessment of Degradation Pathways Based on Physiological Properties

Microbial isolates may be tested for their ability to utilize the parent explosive (i.e., TNT, RDX, or HMX) and any proposed degradation pathway

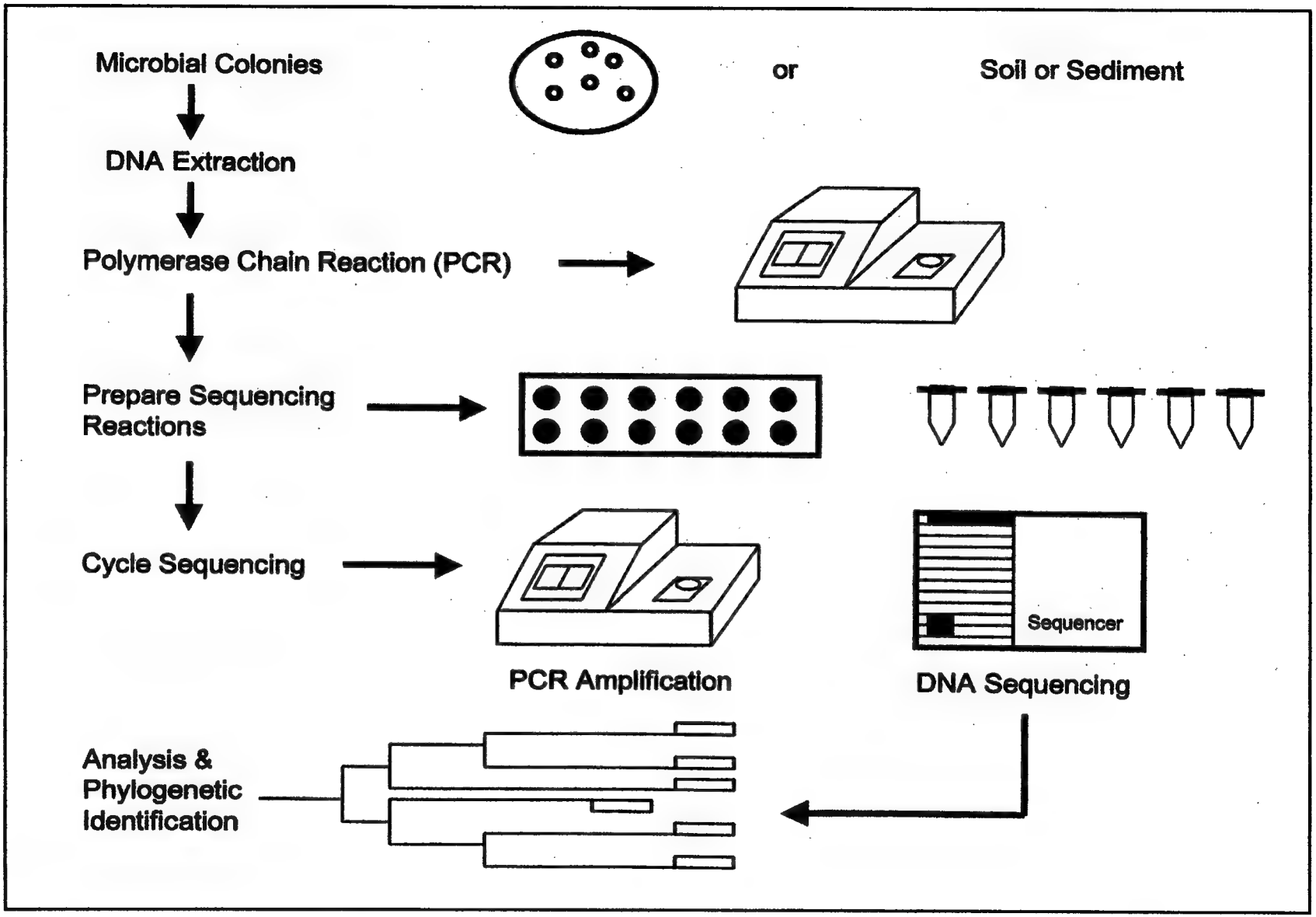

Figure 4. Schematic for bacterial identification using 16S rDNA (modified from http://www2.perkin-elmer.com/pc/MicroSeqProtocol/MicroSeqProtocol.html#Procedure flowchart)

intermediates, sometimes called transformation products, as carbon and energy sources. Those degradation transformation products most likely to be important can often be identified from the refereed literature (see Spain 1995 and Preuss and Rieger 1995 for nitroaromatics; McCormick, Cornell, and Kaplan 1981; Kitts, Cunningham, and Unkefer 1994; and Monpas et al. 1997 for RDX, and Spanggord et al. 1984 for HMX). Degradation of the parent compound can be followed by radioassay as described in Chapter 2; however, many of the potential transformation products (i.e., 2- and 4-aminodinitrotoluenes, the diaminonitrotoluenes, and the azoxy compounds for TNT) and related compounds of interest (i.e., 4-nitrotoluene for TNT) are not available in radiolabeled form. For this reason, classical physiological techniques based on measuring increases in turbidity or changes in respiration resulting from growth on low concentrations of the compound in pure or mixed culture systems are used. The use of a turbidity-based assay allows growth of cultures under anaerobic or microaerophilic atmospheres, if needed. By contrast, a requirement for anaerobic or microaerophilic conditions may limit the utility of a respirometry-based system requiring oxygen. These procedures are widely available (Umbreit, Burris, and Stauffer 1972; Degens and Harris 1997).

The same tests may be conducted using microtiter plates. The technique employs commercially available microtiter plates with sterile mineral salts already in the individual wells to which are added sterile water, suitable sterile carbon sources (i.e., a simple sugar, amino acid, or a degradation pathway intermediate), and the inoculum. Individual wells can be inoculated with pure cultures of microorganisms or suspensions of mixed cultures of microorganisms that have been shown to possess explosives-mineralizing activity. Procedures for working with existing microtiter plate assays are available in Garland and Mills (1991), Zac et al. (1994), or Guckert et al. (1997). Alternate microtiter plate procedures for studying degradation in a soil using radiochemicals are described by Fulthorpe, Rhodes, and Tiedje (1996). Successful growth on the intermediate indicates that the isolate/mixture is able to use the compound. However, repeated transfers of the growing microorganisms from a well containing the proposed intermediate to uninoculated wells containing the same intermediate should be made to ensure that the initial growth of the isolate/mixture was not supported by compounds carried over from the original culture.

Results from growth on intermediates and knowledge of the oxidation-reduction conditions required to support microbial mineralization of the compound activity provide insight into the pathway being used for degradation of the compound.

Assessment of Degradation Pathways Based on Genetic Properties

Modern nucleic acid technologies allow the investigator to detect genes coding for enzymes likely to be used in degradation pathways degrading a given

explosive (Rieger and Knackmuss 1995; Fredrickson et al. 1998). The procedures are summarized in the following paragraph.

Specific degradation (catabolic) genes present within a microbial isolate or a mixture of microorganisms in an environmental sample may be separated from the microorganism(s) using standard microbiological procedures (Sambrook, Fritsch, and Maniatus 1989; Fredrickson et al. 1999) and then identified using either PCR, followed by detection with specific gene probes (Erb and Wagner-Döbler 1993), or by multiplex PCR (Fredrickson et al. 1998). A complete description of the multiplex PCR process, including (a) isolation of DNA from soil or a microbial sample, followed by (b) amplification of targeted bacterial nitroaromatic biodegradation genes, and concluding by (c) assessing the banding patterns diagnostic of targeted genes by slab gel electrophoresis is provided in Pennington et al. (1998) and Fredrickson et al. (1998). In addition, kits are currently available that enable this test to be run under simple conditions in a field laboratory.

Identification of pathways utilized for microbial degradation of an explosive in individual microorganisms or a soil or sediment is most readily accomplished if the pathway(s) is (are) already known. For example, possible pathways for TNT degradation proposed by Rieger and Knackmuss (1995) have been used to select key catabolic enzymes, such as nitroreductases, catechol-2,3-dioxygenases, and biphenyl oxygenases, to monitor during the assessment of natural attenuation of explosives (Pennington et al. 1998).

The techniques used to select key genes for the identification of a catabolic pathway are based on the concept that several key genes can be identified in a sample in sufficient quantities to support the presence of a "degradation pathway." In addition, the relative abundance of catabolic genes can be related to some measure of actual mineralization, such as a radioassay, providing stronger support for the actual use of the pathway for degradation of the explosive in the environment (Pennington et al. 1998). If the possibility for several different degradation pathways is present, then additional information on the environment in which the microorganisms function may support the use of one pathway over another: i.e., is the environment required for degradation activity likely to be aerobic, anaerobic, or switching between the two? Other information required may be obtained by a simple analysis of the materials present in solution form in the soil or sediment. When anaerobic or micro-aerophilic conditions are needed for destruction of explosives, the microbial isolates should be tested for their ability to use alternate electron acceptors, such as nitrate, ferric iron, manganic manganese, and sulfate as replacements for oxygen. In addition, ^{14}C-labeled explosives may be used to determine if carbon from these compounds is metabolized during a fermentation in which ^{14}C-labeled organic acids and alcohols are accumulated. A switch to aerobic conditions may then show whether or not complete mineralization to $^{14}CO_2$ can occur.

While the intermediate and end products for environmental pathways of RDX and HMX degradation were proposed many years ago (McCormick, Cornell, and Kaplan 1981; Spanggord et al. 1984, respectively), the degradation

enzymes for portions of the pathways are just now being established. Recent research has identified one or two enzymes active in RDX degradation (Binks, Nicklin, and Bruce 1996; Young et al. 1997), and these can be selected as target genes for a partial evaluation of an RDX-degrading pathway.

Isolates that utilize pathways easily supported under simple environmental conditions are desirable candidates for further investigation to determine their suitability for research and biotreatment applications. Environmental conditions that can be readily provided include aerobic, anaerobic, or microaerophilic conditions; simple cometabolites (starch, molasses, citrate, glucose, or acetate); alternate electron acceptors (nitrate, nitrite, reduced manganese and iron, sulfate or sulfite, and carbon dioxide); and quiescent or stirred reactor systems. Requirements for excessive heating (above 25-30 °C), large quantities of complex organic compounds (vitamins, cofactors), energy-intensive levels of aeration, or complex inorganic media are not conducive to biotreatment applications.

4 Microbiological and Environmental Restrictions on Explosives Degradation

Use of the procedures described in Chapters 2 and 3 permits the engineer or scientist to obtain explosives-degrading microbial isolates or mixtures of isolates suitable for use in investigations or in biotreatment systems. This chapter indicates the kinds of information that must be obtained to promote the successful survival and activity of the isolate when competition for nutrients, substrate availability, and the ability to outgrow parasites and predators will be necessary attributes for successful research and biotreatment applications.

Relating Microbial Activity and Composition to Function In Situ

The laboratory mineralization assay is limited by the fact that it utilizes freshly added radiolabeled explosive contaminants that have not had the opportunity to age in the environment and may contain low levels (up to 3 percent) of readily degradable impurities. Thus, any mineralization obtained may not accurately reflect what will happen in nature. In addition, the mineralization assay is conducted under ex situ conditions that are often quite different from those present in the environment from which the samples were taken. While the radiolabeled explosives mineralization assay provides a determination of the degradation potential, it does not yield information about how well the laboratory observations predict field results, and how significant the native microorganisms and their catabolic pathways are for explosives degradation under field conditions, how environmental pressures may selectively impact pathway variability, and the rate at which these processes occur. One means for addressing these concerns is by relating the results of laboratory radioassays to the direct analysis of microbial communities with the use of nucleic acid and lipid biomarker techniques. Samples can be examined for the presence of catabolic genes known to participate in the degradation process (catabolic potential) and the microorganism(s) present shown to be member(s) of groups known to degrade the explosives through the presence of characteristic

lipid signatures. These properties, together with the physical and chemical characteristics of the environmental samples (soil texture, pH, presence of contaminants and/or transformation products, nutrient geochemistry), can then be analyzed statistically to determine the ability of measurements made directly on the soil or sediment to predict the rate and extent of mineralization based on the radioassay. If suitable correlations can be developed, reliable predictions may be established describing the ability of environmental samples to support mineralization activity in the field. Several examples of this type of synthesis of information are provided in Pennington et al. (1998).

Assessment of Environmental Considerations for Explosives-Degrading Microorganisms

Basic research investigations are performed to establish the environmental conditions supporting the highest degradation activity. This information is then available for use at the applied bench-scale (6.2) level in determining the type of biotreatment system (or in the case of natural attenuation, no system) that will provide the environmental conditions supporting the most effective type of degradation processes for a given soil.

Basic processes relevant to microbial addition

When an individual microorganism or microbial consortium, whether isolated from the environment (Regen and Crawford 1994; Binks, Nicklin, and Bruce 1995; Crawford 1995; Boopathy and Manning 1996), obtained from commercial sources, genetically engineered (Duque et al. 1993), or enhanced from natural populations through the forced molecular evolution process (described in the next section), is proposed for use in destroying explosives in an environmental matrix, it is important to develop a detailed understanding of the ecology of the organism (interaction with its environment). To assess the ability of an exotic microorganism to perform in the soil or sediment, the following information about the exotic microorganism must be obtained: (a) the ability to grow and carry out explosives degradation activity in the soil or sediment environment, as opposed to a laboratory flask or fermenter; (b) the ability to survive in an environment potentially containing microbial parasites or predators hostile to the added microorganism; (c) the ability to compete successfully in an environment where native microorganisms may be better adapted to take up and utilize other necessary nutrients and/or are better able to tolerate the other contaminants and toxic transformation products present; and (d) the ability to extract and degrade the target contaminant when it is tightly bound to soil components. Obtaining an understanding of these basic ecological properties to enhance the activity of the exotic microorganism is different from applied biotreatability studies wherein a variety of different treatment processes and/or amendments are added to accelerate biodegradation. Knowledge of the ecology of the microorganism is then used to develop specific procedures/processes to enhance the degradation process carried out by the microorganism. Under some circumstances, this type

of approach is preferable to using more expensive procedures to shift the physiological processes conducted by the resident microbial community in hopes that faster and more complete destruction of the explosives can be obtained.

Modification of the soil environment

The soil or sediment environment may sometimes be modified through various chemical and physical means to obtain conditions favoring the growth and activity of the exotic microorganism over competing members of the native microbial community. The exotic microorganism may have specific pH, nutritional, or temperature requirements that can be met in a cost-effective manner. If these requirements are different from those of the native microorganisms, the exotic microorganism may compete successfully with the native microorganisms for nutritional resources required to carry out explosives destruction. The exotic microorganism may then be able to outgrow destructive parasites/predators and be effectively employed for biotreatment (Zappi et al. 1993).

Bioavailability

The term bioavailability refers to the access of the microorganism(s) to the explosive. If the soil binds the explosive to a point where the compound is no longer free to move, the binding process strongly opposes effective destruction of the explosive. However, at the same time, the bound explosive may be unable to exert a toxic effect on the surrounding microflora. Viewed in the light of toxicity reduction, binding may be a perfectly acceptable alternative to microbial degradation. If the ability to place the explosive back into solution is necessary to achieve microbial degradation, then (a) genetic engineering of the exotic microbe to add a surfactant-producing capability, (b) addition of a different, but compatible, exotic microorganism that produces a surfactant, or (c) addition of a man-made surfactant may be necessary. On occasion, explosives-degrading isolates that also produce a suitable surfactant are obtained, but here the level of surfactant production may not be high enough to sustain acceptable removal rates for the explosive. One example of this type of isolate is the isolate *Pseudomonas aeruginosa* G52, which was found to carry out low levels of TNT degradation on its own. However, this microorganism was much less effective at degrading TNT than at producing a surfactant discovered by Dr. Joanne Jones-Meehan, presently at the Naval Research Laboratory.[1] When this microorganism was added to a culture together with either *Ps. fluorescens* or *Enterobacter gregoviae,* isolates obtained from the same soil, the performance of both of the cultures containing the *Ps. aeruginosa* was markedly enhanced, sometimes resulting in total mineralization levels of 6 percent in 2 weeks (Figure 5).

[1] Personal Communication, February 1999.

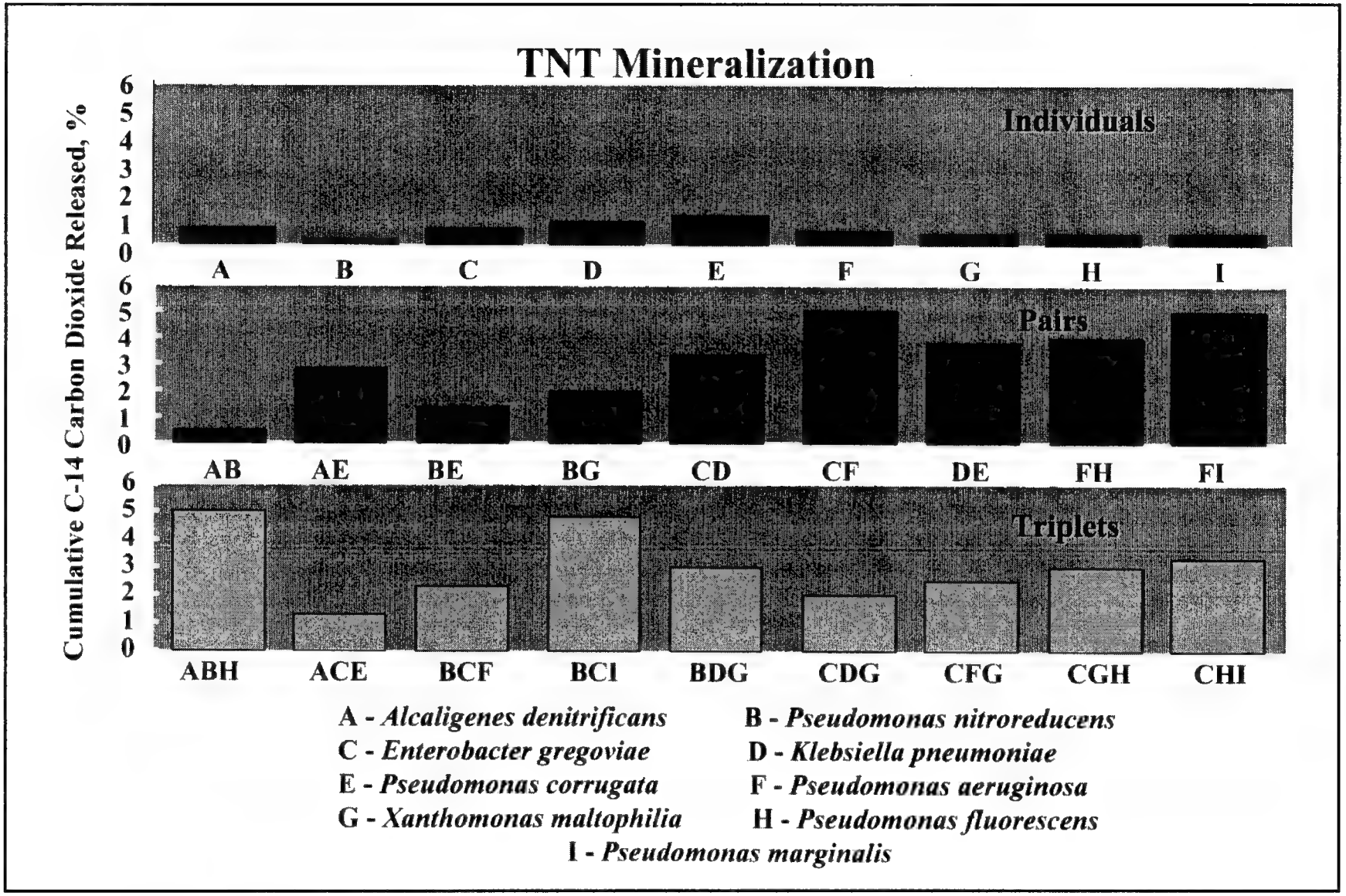

Figure 5. Multiple combinations of isolates and their ability to mineralize TNT. Extent of mineralization was determined by radioassay over a 2-week incubation period. Isolates obtained from Hastings East Industrial Park soil

Assays to establish limiting factors under ex situ conditions

Much of this effort becomes an attempt to verify the activity of the microbial isolate under conditions possessing the major environmental properties of the environment in which the isolate will be used as an exotic species. In this case, the bioslurry and biocell systems described by Zappi et al. (1993) and by Gunnison, Pennington, and Zappi (1996) can be used. However, instead of relying on the native microflora for destruction of the explosive compound(s), an explosives-degrading microorganism or microbial community selected by the procedures described under Tiers I and II of Gunnison, Pennington, and Zappi (1996) is added to the system. The exact level of microbial addition for application is based on the weight of soil to receive the amendment, and normally may run between 0.001 and 0.01 percent of the weight of soil in the system. The system is established in a manner designed to give the exotic microorganism the advantage over native microorganisms. Treatments used may include additional nutrients, use of a cometabolite, and use of either a man-made surfactant or a surfactant-producing second exotic microorganism, such as those described in the preceding paragraph.

Activity Enhancement: Forced Molecular Evolution versus Mass Culture and Addition

On occasion, the optimum destruction of the target compound may require the presence of a microbial community having a large variety of microorganisms, each contributing to the overall genetic capability of the community. If time permits, it may be possible to enhance the activity of the microbial community by addition of a combination of (a) a cometabolite; (b) one or more compounds having structural similarity to the target explosive, but which are simpler and easier to use (example, mono- or dinitroaniline for TNT); and (c) a gradually increasing titer of the target explosive. Through gradual changes in the levels of these compounds over time, it may be possible to enhance the activity of the microorganisms against the target explosive, while at the same time diminishing the number of different microorganisms involved through the process of gene transfer (Gunnison et al. 1997).

5 Application to Passive and Active Biotreatment Scenarios

Investigations leveraged with research support from this work unit included work units on the use of explosives and biomarkers in the fate and effects area of the Research, Development, Tools, and Engineering Program and under several other reimbursable studies. Application of this technology to different biotreatment systems is described in Zappi, Gunnison, and Fredrickson (1995); Best et al. (1998); and Pennington et al. (1998). Examples of 6.2-level projects in which the information developed in the basic supporting microbiology work discussed in previous chapters was used are described in this chapter.

Natural Attenuation

The application of biomarkers to determine the potential for in situ microbial communities to attenuate explosives, as described in Pennington et al. (1998), was leveraged with basic supporting research funds because of the fundamental nature of the processes examined. These methods are summarized and cross-referenced in Chapter 4. The same techniques for identifying microbial communities active in contaminant degradation, delineation of individual microorganisms, and detection of the presence of genes catalyzing degradation processes active in the removal of a contaminant of interest can also be applied to monitoring active biotreatment processes that utilize native microorganisms, such as phytoremediation, land-farming, and in situ treatment technologies. Additional factors that should be considered for biocell, land-farming, and in situ biotreatment applications are examined in Gunnison, Pennington, and Zappi (1996).

Rhizosphere-Enhanced Phytoremediation

A microbial consortium obtained from the 1.5-year-old forced molecular evolution culture (Gunnison et al. 1997) was added as a treatment amendment to

a continuous-flow laboratory phytoremediation study containing the submersed plants *Ceratophyllum demersum* L. (coontail), *Potomogeton nodosus* Poir. (American pondweed), the emergent plant *Sagittaria latifolia* Willd. (Common arrowhead) and to sediment-only controls (Best et al. 1998). The consortium contained a 2,6-DNT-degrading *Sphingomonas* bacterium and an unidentified fungus and was grown on a medium containing 100 mg 2,6-DNT per liter. After addition to the laboratory systems, it was effectively diluted to 0.1 of the original culture. The plants were examined for their ability to phytoremediate explosives-contaminated groundwater from the Iowa Army Ammunition Plant, Middleton, IA. Rates of RDX decrease were found to be significantly greater in planted than unplanted sediment treatments, but the microbial amendments significantly enhanced RDX disappearance. Sediment amended with microorganisms removed 53 percent of the original 10,698 ($\mu g \cdot L^{-1}$) RDX content in 49 days, while coontail treatments amended with the microbial consortium removed 80% of the same RDX concentration in 49 days (Best et al. 1998).

Groundwater Biotreatment

Application of natural microbial communities for in situ biotreatment of groundwater has been studied for RDX and TNT using a modification of the radiorespirometry procedure initially developed under this 6.1 work unit. Details of RDX biotreatment in the aquifer underlying Cornhusker Army Ammunition Plant, Hall County, Nebraska (Waisner 1998), were examined under the Environmental Quality and Technology (EQT) program and Strategic Environmental Research and Development Program (SERDP). The successes of the 6.2 bench- and pilot-scale testing were subsequently proposed as a field demonstration project applying a funnel and gate approach to create anaerobic and aerobic zones in the aquifer following delivery of the requisite amendments through wells upstream of the treatment zone (Waisner 1998).[1] Groundwater biotreatment of TNT under in situ conditions is also being examined using newly emerging technologies in lipid and bioflow cell approaches supported by this 6.1 work unit and by 6.2 EQT work unit in in situ biotreatment.[2] Groundwater biotreatment with the use of microbial isolates having affinities for specific explosives (examples: 2,4- and 2,6-dinitrotoluene) is being actively pursued under the SERDP Flask-to-Field Program by Dr. Jim C. Spain of Tyndall Air Force Base. Dr. Spain is using a granular activated carbon bed as a growth substrate for his microbial isolate in a fluidized bed reactor being used to treat

[1] Specific details of this work may be obtained from Scott A. Waisner at the U.S. Army Engineer Waterways Experiment Station (WES), Vicksburg, MS, phone 601-634-4820.

[2] Detailed information on these processes may be obtained from Dr. Herb Fredrickson, WES, at 601-634-3716 or Dr. Douglas Gunnison, WES, at 601-634-3873.

2,4- and 2,6-DNT-contaminated groundwater at the Volunteer Army Ammunition Plant National Test site, near Chattanooga, TN.[1]

Bioslurry Treatment

Aerobic bioslurry treatment of explosives-contaminated soils has been conducted for Hastings East Industrial Park (Zappi, Gunnison, and Fredrickson 1995). While basically a 6.2 bench-scale application, the basic research forming the foundation for the microbial component of explosives bioslurry treatment was conducted under the basic supporting microbiology work unit. The results demonstrated disappearance of TNT, followed by formation and disappearance of aminonitrotoluenes (monoaminodinitrotoluenes and diaminomononitrotoluenes) and nitrobenzenes (di- and trinitrotoluenes). The authors observed that bioslurry systems had much faster removal kinetics than the bioagricultural (land farming) systems due to vastly enhanced mass transfer in the bioslurry system. Similar bioslurry investigations have been conducted successfully using exotic microorganisms discussed previously using other contaminants (Zappi et al. 1993; Zappi et al. 1996).[2] Bioslurry investigations of TNT under alternating aerobic/anaerobic conditions have been examined with Joliet Army Ammunition Plant (Manning, Boopathy, and Kulpa 1995) soils. The U.S. Army Engineer Waterways Experiment Station assisted in this effort by evaluating the use of man-made surfactants in enhancing the degradation effort under the basic supporting microbiology research program. These efforts have demonstrated that TNT can be removed very effectively under aerobic/anaerobic switching conditions.[3]

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[1] Specific details on this project may be obtained from Dr. Rakesh Bajpai, Acting Director, Federal Integrated Biotreatment Research Consortium, WES, phone 601-634-4823.

[2] Details of the bioslurry process can be obtained from Dr. Gunnison or Professor Mark Zappi at the Department of Chemical Engineering, Mississippi State University, 601-325-7203.

[3] For details of the work at Joilet, contact Mr. Mark Hampton, U.S. Army Environmental Center (USAEC), phone 410-612-6852.

6 Summary

The presence of explosives-degrading microorganisms in soil or sediment samples may be established through a simple screening test that indicates visual presence of activity by growth, clearing of the explosive around the periphery of the test sample, and/or coloration of the media following incubation under aerobic, anaerobic, or microaerophilic conditions. Based on positive outcomes from the screening test, soils having active microbial populations are selected and tested for explosives-degrading activity using a radioassay to establish mineralization of the explosives and by identification of known transformation products in culture media. Soil and sediment possessing microbial activity able to mineralize 10 to 15 percent of the explosive in 28 to 30 days are suitable for use in isolating individual and/or mixtures of microorganisms. Isolates are then characterized by their lipid and/or 16S rDNA content. The explosives degradation pathway(s) employed by the isolate(s) are identified by establishing the ability to grow on major pathway intermediates and by establishing the presence in the isolate(s) of catabolic genes needed for the pathway. Isolates considered desirable candidates for possible use in research or biotreatment applications are tested to (a) determine if they will grow and carry out explosives degradation as exotic microorganisms in contaminated soil or sediment; (b) determine if simple treatments of the soil or sediment will enable the exotic microorganism to compete successfully with native microbial communities; and (c) establish the need for addition of other microorganisms and/or surfactants to improve the bioavailability of the explosive for degradation. Microbial isolates shown to be successful through these tests can be applied to a wide variety of research and biotreatment applications.

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13. ABSTRACT ***(Maximum 200 words)***

This report pulls together past and recently completed research efforts conducted for the 6.1 basic work unit on "Microbiology and Degradation of Explosives." Results are summarized in the form of guidance on the isolation and physiological, biochemical, genetic, and environmental characterization of explosives-degrading microorganisms. Primary emphasis is placed on those microorganisms degrading 2,4,6-trinitrotoluene (TNT) and 2,4- and 2,6-dinitrotoluene (2,4- and 2,6-DNT), but microorganisms degrading hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX or cyclonite), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) are also considered. The first section describes methods used to determine the presence and characteristics of explosives degradation. Topics covered include a method for screening soils for the presence of explosives-degrading activity, application of radiorespirometry to assess the ability of the microorganisms to degrade explosives to $^{14}CO_2$ and other products, and extraction and identification of explosives and explosives degradation products in samples from soil and water slurries incubated with explosives. The second section examines procedures used to evaluate the physiological, biochemical, and genetic properties of explosives-degrading microorganisms. Here, procedures used for isolation of these microorganisms are described and the application of modern lipid and nucleic acid technologies for identification of microbial isolates are presented. This section also summarizes the use of physiological and genetic

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properties to determine the degradation pathways utilized by microbial isolates and groups of isolates. The third section discusses microbial and environmental restrictions on explosives degradation. Particular emphasis is placed on relating microbial activity and composition to function in situ and assessing environmental considerations for explosives-degrading microorganisms, with an emphasis on (a) basic processes relevant to microbial addition, (b) modification of the soil environment to favor addition of explosives-degrading microorganisms, (c) assessment of bioavailability, and (d) determination of limiting factors under ex-situ conditions. The third section also considers the enhancement of microbial activity through use of forced molecular evolution versus use of mass culture and addition of native microbial species. The last section considers practical application of the basic procedures developed in the 6.1 work unit to line-funded and reimbursable biotreatment projects at the 6.2 level. Biotreatment scenarios examined include natural attenuation, rhizosphere-enhanced phytoremediation, and bioslurry treatment.